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Purification and identification of a nuclease activity in embryo axes from French bean

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ABSTRACT

Plant nucleases are involved in nucleic acid degradation associated to programmed cell death processes as well as in DNA restriction, repair and recombination processes. However, the knowledge about the function of plant nucleases is limited. A major nuclease activity was detected by in-gel assay with whole embryonic axes of common bean by using ssDNA or RNA as substrate, whereas this activity was minimal in cotyledons. The enzyme has been purified to electrophoretic homogeneity from embryonic axes. The main biochemical properties of the purified enzyme indicate that it belongs to the S1/P1 family of nucleases. This was corroborated when this protein, after SDS-electrophoresis, was excised from the gel and further analysis by MALDI TOF/TOF allowed identification of the gene (*PVN1*) that codes this protein. The gene that codes the purified protein was identified. The expression of *PVN1* gene was induced at the specific moment of radicle protrusion. The inclusion of inorganic phosphate to the imbibition media reduced the level of expression of this gene and the nuclease activity suggesting a relationship with the phosphorous status in French bean seedlings.

1. Introduction

Seed germination and post-germinative growth are crucial in the life cycle of higher plants. Seeds accumulate reserve materials such as starches, proteins and lipids during their development on the mother plant. During germination and postgerminative growth, seedlings completely depend on these seed reserves [1]. The mobilization of nitrogen or phosphorous in germinating seeds and seedlings has received much lower attention than carbon metabolism. In addition to nitrogen, the developing axes demand high amounts of phosphorous for the synthesis of nucleic acids. In fact, the availability of nucleotides during the early stages in seedling development is critical for the germination success [2]. Nucleotides also participate in many bioenergetic processes required for reserves mobilization during postgerminative growth. Therefore, there is a strict regulation between the synthetic, salvage and degradation pathways of nucleotides during germination and postgerminative growth [3]. The nucleotides may be synthesized de novo or from nucleobases and nucleosides via salvage pathways [4]. During the first stages in germination, the nucleotides are believed to be obtained from nucleobases and nucleosides via salvage pathways with an important energetic advantage [3]. Nucleotides synthetized de novo acquire relevance with the progress of germination, since they are highly required for nucleic acids synthesis in the dividing cells [1].

A source for these nucleotides could be nucleic acids stored in seeds, which could be considered as a potential valuable phosphorous storage compounds. This is particularly true for species with large genomes, and for many plants with seeds that contain high DNA content as result of endoreduplication, a process during which the nuclei undergo repeated rounds of DNA replication without mitosis. *Phaseolus vulgaris* seeds contain endopolyploid nuclei in cotyledons and embryonic axes [5]. The precise physiological function of endoreduplication is still debatable. Nucleic acids are also present in dried seeds as RNA [6], which may be used transiently during early germination for protein synthesis. As germination proceeds, RNA is replaced and protein synthesis becomes more dependent on the new transcripts [1]. The nucleic acid content in dry seeds might be, as well, a reserve to supply material for nucleic acid synthesis in developing axis. Therefore, nucleic acids components might represent an additional nitrogen reserve. In French bean, a phosphatase activity with high affinity for nucleotides is induced during postgerminative growth [7] in parallel with an accumulation of ureides and an induction of its metabolism [8].

The enzymes that catalyze the hydrolysis of the phosphodiester linkage present in nucleic acids are the nucleases [9]. Plant nucleases are usually classified according to their metal ion cofactors and

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their optimum pH [9]. The first class is Zn^{2+}-dependent endonuclease characterized by the requirement for Zn^{2+} and by an acid optimum pH. This group of enzymes preferentially use RNA and ssDNA rather than dsDNA as substrates [9]. The second class is Ca^{2+}-dependent endonuclease characterized for the requirement of Ca^{2+}, by a neutral optimum pH and for its active site similar to staphylococcal nucleases [10]. In plants, extensive hydrolysis of DNA occurs during programmed cell death (PCD) [9]. This process allows remobilization of nutrients from the dying tissues to the other plant tissues, such as storage organs or expanding tissues. Some authors suggested that hydrolysis of nucleic acids during PCD provides large amounts of nucleotides, nitrogen and phosphorous to form the new tissues [11]. The involvement of PCD during development and pathogenesis has been reviewed recently [12]; although relatively little is known about PCD in plants in relation with animals. However, recent studies have compared PCD in plants and animals [12–14]. PCD plays an essential role in the processes of development and germination in cereal grains, and they has been proposed as a model system for the study of PCD in plants (discussed in [15]). An acidic endonuclease involved in nucleus dismantling has been identified in scutellum of wheat grains following germination [15]. Endonuclease activities have been detected as well in growing part of developing wheat seedlings [16]. The crucial involvement of nuclease activities in germination and postgerminative growth can be deduced as well from the complete study performed in cauliflower seedlings, where a total of 14 different nucleases has been identified [17].

In this paper, we present the biochemical characterization of an enzyme showing nuclease activity during French bean germination. We report here the identification and purification of an enzyme responsible for the major activity detected in whole embryonic axes, which is induced after the radicle emergence.

2. Materials and methods

2.1. Plant material and treatments

Common bean (*P. vulgaris* L. cv. Great Northern) seeds were a generous gift from Prof. A. De Ron (Misión Biológica de Galicia, CSIC, Pontevedra, Spain). Seeds were surface-sterilised in ethanol (30 s) and 0.2% (w/v) sodium hypochlorite (5 min) and then washed thoroughly with distilled water. The seeds were allowed to germinate in Petri dishes (120 mm diameter) with paper soaked with water in sterile conditions. When indicated, 5 mM phosphate was added to water. The moisture was maintained by regularly adding solutions to the dishes.

2.2. Preparation of crude extracts

Plant material was homogenized in a chilled mortar with a pestle by adding 5 ml of extraction buffer (50 mM TES pH 6.5 and 0.15% (w/v) sodium deoxycholate) per gram of tissue. The homogenate was centrifuged at 22,000 × *g* for 10 min at 4 °C and the supernatant was considered as crude extract.

2.3. Purification

All purification steps were conducted at 4 °C in working buffer (50 mM TES pH 6.5).

2.3.1. Ammonium sulphate fractionation

Crude extracts were brought up to 65% saturation with ammonium sulphate by stepwise addition of the salt. After gentle stirring for 30 min, the suspension was centrifuged at 22,000 × *g* for 15 min. The supernatant fraction was recovered and brought to 90% ammonium sulphate saturation, stirred and centrifuged as above. The resulting pellet was resuspended in the minimal volume of working buffer.

2.3.2. Heat treatment

The above pool was dialysed overnight against working buffer supplemented with 5 mM $ZnCl_2$. The dialysed preparation was heated at 60 °C for 30 min. After cooling down to 4 °C for 20 min, the solution was centrifuged at 22,000 × *g* for 10 min.

2.3.3. Anion-exchange chromatography in diethylaminoethyl (DEAE)-Sephacel

The supernatant was loaded onto a DEAE-Sephacel column (1 ml, GE Healthcare) previously equilibrated with working buffer and coupled to an AKTA purifier HPLC. The column was washed with 5 column volumes of working buffer, and proteins were eluted using 30 column volumes of a linear gradient of 0–0.5 M NaCl in working buffer at a flow rate of 1 ml min^{-1}. Finally, 5 column volumes of working buffer supplemented with 1 M NaCl was applied. Fractions of 1 ml were collected and further analyzed for nuclease activity.

2.3.4. Affinity chromatography in Concanavaline-Sepharose

The fractions with nuclease activity were pooled and subjected to ConA-Sepharose 4B column (1 ml, GE Healthcare) coupled to an AKTA purifier HPLC. The chromatography was performed at a flow rate of 0.5 ml min^{-1}. The column was previously equilibrated with working buffer supplemented with 1 mM $CaCl_2$, 1 mM MnCl and 0.5 M NaCl. The column was then washed with 5 column volumes of the same buffer. Bound proteins were eluted with a gradient of 0–0.5 M methyl α-D-mannopyranoside in 30 column volumes, followed by a wash of 8 column volumes of 0.5 M of sugar. Fractions of 1 ml were collected.

2.4. Enzymatic activities

2.4.1. In gel nuclease activity assay

In gel-nuclease activity assay was based on the method of Yen and Green [18] with some modification.

Standard ssDNA assay. The resolving mini gels contained 0.24 mg ml^{-1} salmon testes DNA (Sigma–Aldrich) boiled for 5 min immediately prior to pouring the gel. Equal amounts of protein or fresh weight were incubated for 10 min at 40 °C in sample buffer (0.125 M Tris pH 6.8, 10% [v/v] glycerol, 2% [w/v] SDS, 0.01% [w/v] bromophenol blue). In some tests, DTT was added to sample buffer. Electrophoresis were performed at 4 °C, applying 100 V. After electrophoresis, the resolving gels were washed twice at room temperature in 0.01 M acetate buffer pH 5.5 containing 2-propanol (25%, v/v) for 10 min to remove SDS. Subsequently, the gels were washed twice at 4 °C in 0.01 M acetate buffer (pH 5.5). The activity was developed at 50 °C in 0.1 M acetate pH 5.5 for a variable time (5–30 min). The gel was stained with Toluidine Blue O (0.2%, Sigma–Aldrich). The gels were photographed using a UviDoc system.

RNA assay. The activity was determined as above but the resolving mini gels contained 0.75 mg ml^{-1} ribonucleic acid from torula yeast (Sigma–Aldrich) replacing DNA.

Neutral ssDNA assay. The assays were performed as above but using 0.01 M Tes buffer instead of acetate buffer.

In gel-assay in the presence of cations. The assay were performed as above in buffer containing several cations at a final concentration of 1 mM.

2.4.2. In vitro nuclease activity assay

In vitro-nuclease activity assay was based on the method of Wood et al. [19] with some modification. Salmon testes DNA

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